SCREENING AND CHARACTERIZATION OF TOXIGENIC *ASPERGILLUS* SPECIES DIVERSITY IN CATTLE FEED AND AFLATOXIN CONTAMINATION IN DAIRY PRODUCTS IN AND AROUND JAIPUR, RAJASTHAN, INDIA

Synopsis

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CERTIFICATE

It is certified that Mrs. Divya Sarabhai is submitting her synopsis for Ph.D. under my guidance on the Topic 'SCREENING AND CHARACTERIZATION OF TOXIGENIC *ASPERGILLUS* SPECIES DIVERSITY IN CATTLE FEED AND AFLATOXIN CONTAMINATION IN DAIRY PRODUCTS IN AND AROUND JAIPUR, RAJASTHAN, INDIA.'

She has prepared this synopsis under my guidance and supervision and I have gone through the content presented in this report.

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INDEX

CONTENTS	PAGE NO.
1. ABSTRACT	1
2. INTRODUCTION	2
3. REVIEW OF LITERATURE	3
4. DESCRIPTION OF BROAD AREA/ TOPIC	7
5. JUSTIFICATION FOR SELECTION OF RESEARCH AREA AND TOPIC	7
6. OBJECTIVES OF THE STUDY/ PROBLEM IDENTIFICATION	7
7. METHODOLOGY TO BE ADOPTED	8
8. PROPOSED OUTCOME OF THE RESEARCH	10
9. LIMITATIONS OF RESEARCH WORK	11
10.WORK PLAN/TIME LINES	12
11.REFERENCES	13

Abstract

Cattle feed contaminated with fungal species shows the presence of toxins produced by fungi called mycotoxins. Such fungal species are known as toxigenic fungi. These mycotoxins are basically secondary metabolites that have several harmful effects. But all fungi do not produce these toxins and are hence non-toxigenic in nature. Among mycotoxins, Aflatoxins are the most dangerous. They are a group of mycotoxins produced by a mold species called *Aspergillus.* It was first classified by the Italian biologist Pier Antonio Micheli in 1729 (Etymologia: *Aspergillus*, 2006).

Once cattle consume this mycotoxin laden feed, the toxins reach in their body organs and also contaminate their milk produce. When this milk is supplied to the industries and milk factories these mycotoxins become a part of a large variety of other dairy products. Eventually these mycotoxins find their way to the human body where they become a cause of a host of diseases and illnesses like cancer, bronchial irritation, allergies, growth impairment and immune deficiencies (Navale *et al*., 2021).

Keywords: *Mycotoxins, toxigenic fungi, Aspergillus, Aflatoxin, secondary metabolites, dairy products, cancer, allergies, growth impairment, immune deficiencies.*

Introduction

The incidence of mycotoxin contamination in cattle feed has been an issue of concern since the discovery of toxigenic fungal species. The farm cattle usually consume roughage, concentrates, and preserved feeds, which can be a source of many mycotoxins. Many mycotoxins are usually deactivated by the gut microflora of the cattle, but some pass unchanged or are converted into biologically active metabolites. The barrier function of the rumen mostly governs the tendency of cattle towards various mycotoxins. Any damage to this barrier function as a result of a diseases or direct antimicrobial effect of certain mycotoxins, may increase their rates of absorption which not only determines the internal dose and adverse health effects, but also the release of mycotoxins and the biologically active mycotoxins into milk (Fink-Grammels, 2008).

The most dangerous category of mycotoxins is the Aflatoxins. Morphologically, the appearance of this fungus resembled the shape of an aspergillum (holy water sprinkler), hence the name *Aspergillus* (Etymologia: *Aspergillus*, 2006). It is an asexually reproducing fungus, with a spore forming structure known as aspergillum common to all members of the species. Many species of *Aspergillus* cause fungal infections through mycotoxins, while some are known to be commercially important as well (Bennett *et al*., 2010).

A. fumigatus and *A. flavus* are the common aflatoxin, producing pathogenic species of *Aspergillus*. Their toxins are carcinogenic and contaminate both cattle feed as well as agricultural produce. There are some species of *Aspergillus* that cause allergies, like *A. fumigatus* and *A. clavatus*. Various members of this species are also known to cause diseases in grain crops and neonatal infections in humans (Cloherty *et al*., 2012).

In natural food chains, the original mycotoxin is converted into other toxic complexes, while being consumed by the consumer. The incidence of aflatoxin M1 in dairy products is because of existence of aflatoxin B1 in the animal feed. Consequently, milk-based foods can be contaminated with this toxin (Vaz *et al*., 2020).

Molds infect dairy cattle and cause mycosis. Mycosis affects cows when they are immuno-suppressed in stressful periods. It affects organs like lungs, mammary glands, uterus and intestine. Infection in the intestine often results in a haemorrhagic bowel (Carlson and Ensley,

2019). Being present in the mammary glands, these toxins are obviously passed into the milk. When such contaminated milk from a diseased cow is consumed by humans, these toxins are passed to the human body. Although commercially available milk is processed and purified in milk factories and packaging plants, the processes fail to rid this milk of mycotoxins.

Another problem with aflatoxins is that they have been found to be thermostable and resistant to pasteurization (Variane *et al*., 2018). Humans cannot sense their presence by smell or taste and they are not degraded by any bio-chemical process in human or animal bodies (Claudious *et al*., 2019).

Very little research work has been carried out in the Rajasthan on this fungus, where milk and dairy products like chhaas are consumed by the resident population in large quantities. If these products are contaminated with mycotoxins, then it is a serious issue concerning the health of the general population. This research will focus on the estimation of aflatoxin contamination in cattle feed and in dairy products in and around Jaipur city.

Review of Literature

India is the largest and premier producer, as well as consumer, of dairy products in the world. Despite having no subsidy from the government, the dairy industry has shown a remarkable growth in our country. The small farmers with 10 to 20 cattle heads each, are the most crucial and primary source of raw materials for this industry. Cattle farming in India has been a prevalent practice in rural as well as some urban areas. People domesticate cows and buffaloes and breed them for production of milk, for commercial purpose. The farmers cater to the rearing and caring of these cattle.

These small farmers are native of the rural India and due to lack of resources, knowledge and awareness, they are sometimes unable to provide safe and hygienic conditions to their cattle. Several research studies have shown that due to poor storage conditions i.e. improper temperature and pH, the agricultural produce becomes contaminated with toxigenic fungus. All these factors are predominant in India and increase the risk of cattle feed to become infested with toxigenic fungal species (Sheikh-Ali *et al*., 2014).

Once thought of being plants, fungi have emerged as their own taxonomic kingdom. The fungal species show a vast diversity and with many unique properties: some are beneficial and some

harmful. Fungi are eukaryotic, heterotrophic organisms, both uni- and multicellular. Thousands of different fungi are found in the surrounding environment, and humans are constantly exposed to fungi through air, food and water. Most fungi produce chemicals called secondary metabolites that can be both toxic and non-toxic in nature. The toxic secondary metabolites fall under the category of mycotoxins and the fungal species producing them is termed as toxigenic fungus. Toxigenic fungi like, *Aspergillus flavus, Aspergillus parasiticus, Aspergillus oryzae, Aspergillus arachidicola, Aspergillus parvisclerotigenus, Aspergillus alutaceous and Aspergillus nidulans* are some of the fungal strains that are known to be present in cattle feed and produce several harmful mycotoxins (Sheikh-Ali *et al*., 2014).

Aflatoxins are basically Di-furanocoumarin derivatives with a bifuran group at one side of the coumarin nucleus and a pentanone ring on the other side (Bennett and Klich, 2003; Nakai *et al*., 2008). Their biosynthetic pathway has 18 enzyme-catalysed steps and these enzymes are encoded by around 25 different genes (Yu *et al*., 2002). *Aspergillus* sp. is the major source of aflatoxins. It is the mycotoxin that most commonly harm human and animal health (Boutrif, 1998). Toxicity levels associated with aflatoxins varies among different types and is in the increasing order from aflatoxin G2, aflatoxin B2, aflatoxin G1 and aflatoxin B1 (Jaimez *et al*., 2000).

In general, aflatoxins occur more in tropical countries because of conditions like unpredictable rainfall patterns resulting from temperature fluctuations. Poor storage, improper harvesting and careless transport are a few factors that contribute to mycotoxin production in agricultural produce. Chronic health risks, like aflatoxicosis, are predominant in India where the diets of people are highly susceptible to be contaminated with aflatoxigenic fungi and aflatoxins. Mycotoxins are known to have nephrotoxic, genotoxic, teratogenic, carcinogenic, and cytotoxic effects as a result of which they toxins may cause liver carcinomas, renal dysfunctions, and also compromise the immune system (Ráduly *et al*., 2020).
Out of many, the first outbreaks of aflatoxicosis was conveyed from western India during 1974 with 106 deaths of native people whose staple food was maize (Krishnamachari et al., 1975). This was the first report that directly implicated aflatoxin in food as a health hazard for humans. (Reddy *et al*., 2007). Indian childhood cirrhosis has also been ascribed to aflatoxin contamination (Reddy *et al*., 2007). An association was also found between aflatoxin contamination in food and hepatomegaly in children, in south Canara district of Karnataka (Reddy *et al*., 2007: Kumar *et al*., 2016).

Another outbreak of toxic hepatitis affecting both humans and dogs, was stated in India during 1974 (Tandon *et al*., 1977; Bhatt and Krishnamachari, 1978; Reddy *et al*., 2007). Heavy transience in chicks in Chittoor district of Andhra Pradesh was reported in 1982 due to aflatoxicosis. An occurrence of aflatoxicosis in commercial poultry farms was also reported in the same district with hundred percent mortality. Cases of aflatoxicosis in Murrah buffaloes from Andhra Pradesh were also reported. Incidence of aflatoxicosis in poultry in Mysore state was first documented in 1966 where 2219 chicks died in a week (Reddy *et al*., 2007).
Many prior surveys conducted in countries across the world have indicated the risk of transfer of AFB1 to AFM1 in diet through milk. Asia, the Middle East, Africa and South America have reported 100% contamination in various food samples (Jiang *et al*., 2021).

Most of the occurrences of aflatoxicoses from aflatoxin contaminated food. Disease outbreaks due to aflatoxins are still a matter of concern in India as long as people keep consuming contaminated food. The strict control of food quality is essential to avoid such incidences (Ráduly *et al.*, 2020).

Mycotoxin contamination in agricultural produce is a serious threat to both cattle and humans. Consumption of foods rich in mycotoxins may cause both acute and chronic toxicity in organisms. Aflatoxin was on the Rapid Alert System for Food and Feed (RASFF) of the European Union in 2008 because of its effects (European Commission, 2009), and the International Agency for Research on Cancer (IARC) later characterised AFB1 as a group I carcinogen for humans (Min *et al*., 2011). In spite of many control procedures, aflatoxin continues to be a major risk to food and agricultural commodities (Kumar *et al*., 2016). Both the World Health Organisation, Department of food safety and Zoonoses, 2018 and Food and Agriculture Organisation, 2019, have issued strict guidelines to solve this global issue. Their scientific advisory committee is responsible for assessing the health hazards of toxins like aflatoxins, ochratoxins etc.

Apart from being a serious threat to the health of animals and humans, many mycotoxins have also shown to reduce productivity in feed and poultry, significant deaths of live-stock and eventually proves that the fungus is a hazard to food resources and overshadows its ability to decompose plant materials. Aflatoxin B1, is a potent liver carcinogen. It has been observed that specific P450 enzymes in the liver convert aflatoxin into aflatoxin-8,9-epoxide, a reactive

oxygen species, and binds either to proteins and cause acute toxicity (aflatoxicosis) or to DNA and induce liver cancer (Wu *et al*., 2011).

Growth of fungal molds and aflatoxin production is generally caused when a plant is under stress resulting from extreme weather, insect damage, storage malpractices, etc. Mycotoxin concentration in corn, before harvesting, have been observed to have resulted from these conditions (Patience and Ensley, 2010).

Since, cattle feed can get contaminated post-harvesting as well, control of this contamination and consequent toxin production depends on storage conditions. *Aspergillus flavus* and *Aspergillus parasiticus* show growth in the temperature range of 10 ° to 40°C (50° to 104°F) a pH range of 4 to 8, and beyond 0.7 aw (Lacey *et al*., 1991). They show toxin production at 25°C temperature, 85% and 90% relative humidity (Bakirci, 2000).

Feeds with more than 12 to 15% moisture show more mold growth. In wet feeds like silage, higher moisture content leads to mold growth if oxygen is also available (Dairexnet, 2019). Because majority of molds are aerobic, high moisture can reduce oxygen availability and help in preventing mold growth. But the most suitable conditions for mold growth and mycotoxin production are usually not the same. For example, the *Fusarium* molds, linked to alimentary toxic aleukia, grow actively at 25° to 30°C without showing much mycotoxin production, but at lower temperatures, they produce large quantities of mycotoxins, even with minimal mold growth (Marsh, 1986). Applications of fungicides in crop fields may reduce mold growth, reducing mycotoxins production. But the stress or shock resulting from the application of fungicide may decrease mold growth and still not reduce the production of mycotoxins (Boyacioglu *et al*., 1992; Gareis and Ceynowa, 1994; Simpson *et al*., 2001).

Since past many decades, struggles to control mycotoxins have been going on during which various physical, chemical and biological approaches have been tried but only biological treatment has shown promising results. With a specific and efficient action, biological control has a positive effect on the environment, food safety and food security (Nesic *et al*., 2021).

Yeast (Li *et al*., 2019), bacteria (Umesha *et al*., 2017; Schatzmayr *et al*., 2006), fungi (Abbas *et al*., 2011; Okoth *et al*., 2018) and enzymes (Loi *et al*.,2017) show the capability to

destroy/adsorb mycotoxins, which increases the safety and quality of crops, animal feed and, ultimately, food of animal origin (milk, meat and eggs) (Nesic *et al*., 2021). Biodetoxification is an emerging strategy for reducing mycotoxins reduction through microbes or their enzymes. They convert the poisonous mycotoxins to non-toxic forms, and are considered safe as they supress the toxic nature of mycotoxins by simple processes like biodegradation and bio-adsorption. Probiotics like *Lactobacillus* and *Bifidobacterium*, also affect toxigenic fungi by competing for resources like living space and nutrition by forming biofilms (Luo *et al*., 2018; Juodeikiene *et al*., 2018) and release of free oxygen radicals (Afshar et al., 2020).

Although biological control of mycotoxins holds a bright future but it is not self-sufficient. It is most successful when implemented with proper agricultural practices, and post-harvest management. All tests should be performed before implementation and the outcomes should be evaluated for evaluating the risks on health of plants, animals and humans as well as on the environment (Nesic *et al*., 2021).

Description of Broad Area/Topic

This research is proposed on the concept of mycotoxin production by toxigenic fungal species in cattle feed. This research will focus on the identification and screening of *Aspergillus* species contamination in cattle feed and the resultant contamination in milk and dairy products like paneer, chhaas, cheese etc. The quality and quantity of the toxins present will also be assessed and compared to the permitted values.

Justification for Selection of Research Area and Topic

Aflatoxin contamination in dairy products is a serious matter of concern. Consumption of milk and dairy by the common people all over the world is very normal but very few people are aware of the threats posed by consumption of contaminated products. Aflatoxins are capable of causing toxicity in humans in several ways which eventually lead to liver damage and immunosuppression. Statistical data from different parts of the world prove aflatoxins to be a potent cause for liver cancer. Rajasthan, although a dry state, also consist of population of humans largely dependent on milk and dairy products. A lot of research has been going on in different parts of the world for control and monitoring of this contamination everywhere but no significant data about the same being done in Rajasthan has been found.

Objectives of the Study

1. To collect samples of cattle feed, raw milk from farms and milk products like processed milk, milk powder, cheese and cottage cheese from markets in and around Jaipur City.

2. Isolation, identification and screening of *Aspergillus* species of fungi on the basis of cultural and morphological characteristics in cattle feed samples.

3. Identification of toxigenic and non-toxigenic *Aspergillus* species of fungi by fluorescence.

4. Genome Analysis of toxigenic *Aspergillus* isolates by 18S rRNA Sequencing.

5. Qualitative analysis of aflatoxin using Enzyme Linked Immuno-Sorbent Assay (ELISA) and quantitative analysis through High Performance Liquid Chromatography (HPLC).

6. Testing the efficacy of control and preventive methods to reduce Aflatoxin levels in cattle feed.

Methodology to be Adopted

1. Sample Collection

The following samples will be collected from sources in and around Jaipur City:

1. Cattle feed samples from dairy farms.
2. Samples of raw, unprocessed milk and processed milk.
3. Collection of milk products from unorganised and organised dairy units.

2. Isolation, Purification and Identification of Toxigenic Fungi from Samples of Cattle Feeds.

The collected samples of cattle feed will be first surface sterilized with sodium hypochloride followed by rinsing with distilled water. Samples will then be placed on Potato Dextrose Agar petri plates and incubated at 25ºC for 5 days. Developed colonies will be purified by single spore or hyphal tip method and then transferred to PDA slants. Isolates observed will be identified by using keys and manuals up-to species level on the basis of morphology and microscopic characteristics (Al-Wadai *et al*., 2013).

Different strains of *Aspergillus* species will be identified on the basis of colony morphology, staining and microscopic characteristics.

3. Identification of Toxigenic and Non-toxigenic *Aspergillus* Species of Fungi by Fluorescence.

After their successful identification as *Aspergillus* species, the strains will be cultivated on PDA slants at 25ºC for 7 days. The mycelial plug produced of each strain will be cultured on petri plates containing the test media and will be incubated at 25ºC for 4 days in dark. The petri plates will then be exposed to 365 nm UV light and will be deemed as toxigenic or non-toxigenic on the basis of presence or absence of fluorescence on the agar surrounding glowing *Aspergillus* species colony (Al-Wadai *et al*., 2013).

4. Genome Analysis of Toxigenic *Aspergillus* Isolates by 18S RNA Sequencing.

The toxigenic fungal isolates will be subjected to DNA Isolation followed by 18S rDNA restriction and sequence analysis (Hunt *et al*., 2004), followed by BLAST. The outcome will be further investigated using MEGA 6.0 software to identify the phylogenetic history of the fungal isolates to construct the phylogenetic tree (Tamura *et al*., 2013).

5. Qualitative Analysis of Aflatoxin Using Enzyme Linked Immunosorbent Assay and Quantitative Analysis Through High-Performance Liquid Chromatography.

- **Sample Preparation:** In order to ensure precision and reliability of results, the collected samples of milk, powdered milk cheese and cottage cheese will first be prepared by centrifugation and filtration (Al-Mossawei *et al*., 2016). These are simple procedures for liquid milk samples and do not require any use of solvents (Vaz *et al*., 2020).

- **Extraction and Clean-up:** This step will ensure the removal of mycotoxins from filtered matrix by use of an appropriate solvent like dichloromethane, chloroform, combinations of polar organic solvents like acetonitrile, acetone and methanol. For cheese samples, organic solvents will be used followed by clean-up. Extraction can also be done by enzymes and their mixtures to obtain liquified extract of solid samples like cheese. The enzymes generally used are pepsin or pepsin-pancreatin mixture (Pietri *et al*., 2016) Clean-up ensures exclusion of matrix interfering and preconcentration of the analyte (Ketney *et al*., 2017). The clean-up is done after extraction for precision and accuracy of outcomes

(Manetta *et al*., 2005). Generally, for the purpose of clean-up, Immuno-Affinity Columns (IACs) are used for liquid milk samples followed by analysis of aflatoxin. These methods do not involve extraction. A single step and multifunctional Mycosep^{TM} can be used for solid samples, after the step of extraction (Vaz *et al*., 2020). Mycosep^{TM} columns use adsorbents packed in plastic tubes to retain any interfering substances and separation of analyte from them (Hussein 2011, Iqbal *et al*., 2015).

- **Quantification:** The quantitative estimation of aflatoxin in dairy samples will be analysed by Liquid Chromatography followed by fluorescence detection (LC-FLD). This method provides high sensitivity, dynamic range and is a versatile technique (Espinosa-Calderón et al., 2011). Recent methods of LC-FLD utilize reverse-phase HPLC methods for separation. To determine aflatoxin levels in milk and cheese samples, HPLC with fluorescence detection will be used. This method is simple and automated, gives accurate and precise values of contamination of aflatoxins in milk and cheeses (Manetta *et al*., 2005).

- Standard curve solutions of AFM1 will be prepared by diluting stock solution with acetonitrile. The mobile phase will be a mixture of acetic acid, acetonitrile, 2-propanol and water in the ratio 2:10:10:78, by volume. HPLC will be performed with an aqueous solution of pyridinium hydrobromide perbromide as a post-column derivatising agent. Identification of aflatoxins will be done on the basis of retention time (Manetta *et al*., 2005).

- Alternatively, rapid methods for screening of mycotoxins in milk samples can also be used. These methods are popular for screening before the chemical analysis. They are immunochemical assays like Enzyme Linked Immunosorbent Assay (ELISA) using fluorometric detection. The detection method is simple, sensitive and adaptable. It has been commonly used for aflatoxin detection in a variety of dairy products. This technique is based on antigen-antibody interactions. Cheese samples require a step of extraction to remove interference by fats (r-Biopharm RIDASCREEN ®, 2022). The technique is performed 384-well micro titre plate with specific monoclonal antibody for capturing and secondary conjugated antibodies for detection and quantification of the analyte. The plate will be covered with parafilm and aluminium foil and incubated at kept at 4°C, overnight. After washing the plates with PBS, the remaining protein binding sites in the coated wells will be blocked by blocking solution followed by incubation at room temperature for an

hour. The wells will be re-washed with PBST. Following this step, antigen-antibody mixture solution will be added in each well followed by incubation at room temperature for two. The microplate will then be analysed by fluorescence reader (Kanungo and Bhand, 2013).

6. Testing of Control Measures

To minimize levels of aflatoxins in milk and dairy products, physical, chemical, and biological control methods for reducing growth of *Aspergillus* in cattle feed will be tested. For testing the efficacy of physical control measures, growth of *Aspergillus* will be observed at various levels of temperature and moisture. For chemical control measures, *Aspergillus* growth at different pH will be observed, and for testing the efficacy of bio-control methods, reduction in growth of *Aspergillus* will be observed in presence of Yeast, *Lactobacillus* and *Bifidobacterium*.

*** Proposed methodology is subject to modification as per requirement of the research problem.**

Proposed/ Expected Outcomes of the Research

This research will help us to identify various species of *Aspergillus* that infect cattle feed and determine the concentration of aflatoxins in various varieties of commercially available milk (raw as well as processed) in Jaipur. The findings of this research will help to monitor the levels of the toxins in milk and determine them as allowable or alarming as per the FSSAI guidelines. According to FSSAI, the standard limit of aflatoxin M1 in liquid milk is 0.5 µg/kg, in skimmed milk powder is 6µg/kg, and in whole milk powder is 4 µg/kg (September 2020). This research may also pave way to estimate the potential health hazards of mycotoxins to human population in Jaipur, Rajasthan.

In addition, as per the results and findings, possible strategies to control, prevent and reduce the contamination of mycotoxins in cattle feed and milk and dairy products will also be suggested.

Limitations of Research Work

The proposed research work is confined only to analyse and determine the types and content of Aflatoxins in various dairy products. The experimentation and methodology is not designed to reduce Aflatoxin levels in milk and other dairy products. However, some aspects of physical, chemical and biological controls will be tested to reduce the incidence of toxigenic fungus in the cattle feed which will only give an outlay of how toxigenic *Aspergillus* species can be mitigated in cattle feed. It is assumed that if any of these control methods prove to be successful and applicable at large scale, the levels of Aflatoxin in milk and dairy products will reduce automatically.

Work Plan/Time Lines

Research Activity	6	12	18	24	30	36	*
1. Course Work							
2. Literature Survey							
3. Methodology Steps							
4. Quarterly Reports							
5. Communication of Papers							
6. Preparation of Final Thesis							
7. Submission of Thesis							

* As approved Extension

References

Abbas, H, *et al*., (2011) 'Comparison of major biocontrol strains of non-aflatoxigenic Aspergillus flavus for the reduction of aflatoxins and cyclopiazonic acid in maize', *Food Additives & Contaminants: Part A*, 28(2), pp.198-208.

Afshar, P. *et al*., (2020) 'Aflatoxins biodetoxification strategies based on probiotic bacteria' *Toxicon*, 178, pp.50–58.

Al-Mossawei, M.T. *et al*., (2016) 'Detection of AFM 1 in Milk and Some Dairy Products in Iraq using different techniques', *Advance Life Science Technology*, 41, pp. 74-81.

Al-Wadai, A.S. *et al*., (2013) 'Molecular characterization of Aspergillus flavus and aflatoxin contamination of wheat grains from Saudi Arabia', *Genetics and Molecular Research*, 12(3), pp.3335–3352.

Bennett, J.W. & Klich, M., (2003) 'Mycotoxins', *Clinical Microbiology Reviews*, 16(3), pp.497–516.

Bakirci, I., (2001) 'A study on the occurrence of aflatoxin M1 in milk and milk products produced in Van province of Turkey', *Food control*, 12(1), pp.47-51.

Bennett, J.W., Machida, M., and Gomi, K. (2010) **'**An overview of the genus *Aspergillus*, In *Aspergillus', Molecular Biology and Genomics*, Caister Academic Press. 1, pp. 1–17.

Bhat, R., Rai, R.V. and Karim, A.A., (2010). Mycotoxins in food and feed: present status and future concerns. Comprehensive reviews in food science and food safety, 9(1), pp.57-81.

Bhatt, R. V., and Krishnamachari, K. A. V. R., (1978), 'Food toxins and disease outbreaks in India', *Arogya Journal of Health Sciences*, 4, pp.92–100.

Boutrif, E., (1998) 'Prevention of aflatoxin in pistachios', *Food Nutrition and Agriculture*, pp.32-37.

Boyacioglu, D., Hettiarachchy, N.S. and Stack, R.W., (1992) 'Effect of three systemic fungicides on deoxynivalenol (vomitoxin) production by *Fusarium graminearum* in wheat', *Canadian Journal of Plant Science*, 72(1), pp.93-101.

Carlson M. P., Ensley S. M., (2019), 'Understanding Fungal (Mold) Toxins (Mycotoxins)', Available at: https://dairy-cattle.extension.org/understanding-fungal-mold-toxins-mycotoxins/ *DAIReXNET*, [Accessed on: 24 October 2021).

Claudious, G., (2019) 'Isolation Of Aspergillus Flavus from Dairy Cattle Feed and Assessment of Aflatoxin M1 In Milk From Small Dairy Farms Around Harare, Zimbabwe', *Advances in Microbiology Research*, 3(1), pp. 1-7.

Cloherty, J.P., Eichenwald, E.C. and Stark, A.R., (2008) '*Manual of Neonatal Care'*, 7th Edition, Philadelphia, Lippincott Williams & Wilkins.

Devegowda G., Sridhar V., Sherasia P. L., (2019) *Aflatoxin: Prevalence and Control in Dairy Feeds and Milk*, Gujarat, National Dairy Development Board, Booklet.

Espinosa-Calderón, A. *et al*., (2011) 'Methods for detection and quantification of aflatoxins. Aflatoxins: detection, measurement and control', *New York: InTech*, pp.109-128.

Etymologia: *Aspergillus*, (2006) *Emerging Infectious Diseases*.12(3):415. Available at: doi:10.3201/eid1203.et1203. (Accessed on 1 November 2021).

European Commission, (2008), *The Rapid Alert System for Food and Feed (RASFF)*, Annual Report, Luxembourg: European Communities, Available at: https://ec.europa.eu/food/safety/rasff-food-and-feed-safety-alerts_en (Accessed on: 15 November 2021).

Fink-Gremmels, J., (2008) 'Mycotoxins in cattle feeds and carry-over to dairy milk: A review.', *Food Additives and Contaminants*, 25(2), pp.172-180.

FSSAI Manual for Mycotoxins Screening, [Online] Available at: https://www.fssai.gov.in/upload/advisories/2020/12/5fca3db8df192Order_Revised_Manual_Mycotoxins_04_12_2020.pdf (Accessed on: 16 November 2021).

Gareis, M. and Ceynowa J., (1994) 'Influence of the fungicide Matador (tebuconazole/triadimenol) on mycotoxin production by *Fusarium culmorum*', *Lebensmittel-Untersuchung-Forsch*, 198, pp. 244-248.

Hunt, J., Boddy, L., Randerson, P.F. and Rogers, H.J., (2004) 'An evaluation of 18S rDNA approaches for the study of fungal diversity in grassland soils', *Microbial Ecology*, 47(4), pp.385-395.

Hussain, I., (2011) 'Aflatoxin measurement and analysis. Aflatoxins–Detection, Measurement and Control', Rijeka, Croatia: InTechOpen, 1, pp.129-146.

Iqbal, S.Z., Jinap, S., Pirouz, A.A. and Faizal, A.A., (2015) 'Aflatoxin M1 in milk and dairy products, occurrence and recent challenges: A review', *Trends in Food Science & Technology*, 46(1), pp.110-119.

Jaimez, J., Fente, C.A., Vazquez, B.I., Franco, C.M., Cepeda, A., Mahuzier, G. and Prognon, P., (2000) 'Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis', *Journal of Chromatography A*, 882(1-2), pp.1-10.

Jiang, Y., Ogunade, I.M., Vyas, D. and Adesogan, A.T., (2021) 'Aflatoxin in dairy cows: toxicity, occurrence in feedstuffs and milk and dietary mitigation strategies', *Toxins*, 13(4), pp.283-306.

Marsh, P. B. (1987) '*Fusarium* Species-Their Biology and Toxicology', *American Journal of Alternative Agriculture*, 2(3), pp. 140–141. Available at: doi: 10.1017/S0889189300001831 (Accessed on: 15 November 2021).

Juodeikiene, G., Bartkiene, E., Cernauskas, D., Cizeikiene, D., Zadeike, D., Lele, V. and Bartkevics, V., (2018) 'Antifungal activity of lactic acid bacteria and their application for

Fusarium mycotoxin reduction in malting wheat grains', Lebensmittel-Wissenschaft und-Technologie (LWT-Food Science Technology), 89, pp.307-314.

Kanungo, L. and Bhand, S., (2013) 'Fluorometric immunoassay for multi-analysis of aflatoxins', *Journal of Analytical Methods in Chemistry*, 2013, pp. 1-8. Available at: https://doi.org/10.1155/2013/584964 (Accessed on: 15 November 2021).

Ketney, O., Santini, A. and Oancea, S., (2017) 'Recent aflatoxin survey data in milk and milk products: A review', *International Journal of Dairy Technology*, 70(3), pp.320-331.

Krishnamachari, K.A.V.R., Nagarajan, V., Bhat, R. and Tilak, T.B.G., (1975) 'Hepatitis due to aflatoxicosis: an outbreak in western India', *The Lancet*, 305(7915), pp.1061-1063.

Kumar, P., Mahato, D.K., Kamle, M., Mohanta, T.K. and Kang, S.G., (2017) 'Aflatoxins: A global concern for food safety, human health and their management', *Frontiers in Microbiology*, 7, pp. 2170-2180.

Kumar, A., Pathak, H., Bhadauria, S. and Sudan, J., (2021) 'Aflatoxin contamination in food crops: causes, detection, and management: A Review', *Food Production, Processing and Nutrition*, 3(1), pp.1-9.

Lacey, J., (1991) *Mycotoxins and Animal Foods* 'Natural occurrence of mycotoxins in growing and conserved forage crops', CRC Press, Boca Raton, Florida, pp.363-397.

Li, X., Tang, H., Yang, C., Meng, X. and Liu, B., (2019) 'Detoxification of mycotoxin patulin by the yeast *Rhodotorula mucilaginosa*', *Food Control*, 96, pp.47-52.

Loi, M., Fanelli, F., Liuzzi, V.C., Logrieco, A.F. and Mulè, G., (2017) 'Mycotoxin biotransformation by native and commercial enzymes: Present and future perspectives', *Toxins*, 9(4), pp.111-142.

Luo, Y., Liu, X. and Li, J., (2018) 'Updating techniques on controlling mycotoxins-A review', *Food Control*, 89, pp.123-132.

Mahato, D.K., Lee, K.E., Kamle, M., Devi, S., Dewangan, K.N., Kumar, P. and Kang, S.G., (2019) 'Aflatoxins in food and feed: an overview on prevalence, detection and control strategies', *Frontiers in Microbiology*, 10, pp.2266-2276.

Manetta, A.C., Di Giuseppe, L., Giammarco, M., Fusaro, I., Simonella, A., Gramenzi, A. and Formigoni, A., (2005) 'High-performance liquid chromatography with post-column derivatisation and fluorescence detection for sensitive determination of aflatoxin M1 in milk and cheese', *Journal of Chromatography A*, 1083(1-2), pp.219-222.

Min, W.K., Kweon, D.H., Park, K., Park, Y.C. and Seo, J.H., (2011) 'Characterisation of monoclonal antibody against aflatoxin B1 produced in hybridoma 2C12 and its single-chain variable fragment expressed in recombinant *Escherichia coli*', *Food Chemistry*, 126(3), pp.1316-1323.

Nakai, V.K., de Oliveira Rocha, L., Gonçalez, E., Fonseca, H., Ortega, E.M.M. and Corrêa, B., (2008) 'Distribution of fungi and aflatoxins in a stored peanut variety', *Food Chemistry*, 106(1), pp.285-290.

Navale, V., Vamkudoth, K.R., Ajmera, S. and Dhuri, V., (2021) 'Aspergillus derived mycotoxins in food and the environment: Prevalence, detection, and toxicity', *Toxicology Reports*, 8, pp.1008-1030.

Navya, H.M., Hariprasad, P., Naveen, J., Chandranayaka, S. and Niranjana, S.R., (2013) 'Natural occurrence of aflatoxin, aflatoxigenic and nonaflatoxigenic *Aspergillus flavus* in groundnut seeds across India', *African Journal of Biotechnology*, 12(19), pp. 2587-2597

Nešić, K., Habschied, K. and Mastanjević, K., (2021) 'Possibilities for the biological control of mycotoxins in food and feed', *Toxins,* 13(3), pp.198-213.

Okoth, S., *et al*., (2018) 'Genetic and toxigenic variability within *Aspergillus flavus* population isolated from maize in two diverse environments in Kenya', *Frontiers in Microbiology*, 9, pp.57-71.

Patience, J.F., Ensley, S.M. and Hoyer, S., (2010) 'Mycotoxin Contamination of Corn: What it is, What it does to Pigs and What can be done about it', *IPIC, University Extension*. Available at: https://www.ipic.iastate.edu/publications/ipic12.pdf (Accessed on: 15 November 2021)

Patyal, A., Gill, J.P.S., Bedi, J.S. and Aulakh, R.S., (2020) 'Occurrence of Aflatoxin M1 in raw, pasteurized and UHT milk from Punjab, India', *Current Science*, 118(1), pp. 79-86.

Pietri, A., Fortunati, P., Mulazzi, A. and Bertuzzi, T., (2016) 'Enzyme-assisted extraction for the HPLC determination of aflatoxin M1 in cheese', *Food Chemistry*, 192, pp.235-241.

Ráduly, Z., Szabó, L., Madar, A., Pócsi, I. and Csernoch, L., (2020) 'Toxicological and medical aspects of Aspergillus-derived mycotoxins entering the feed and food chain', *Frontiers in Microbiology*, 10, pp.2908-2931.

Rao, V.K., Shilpa, P., Girisham, S. and Reddy, S.M., (2011) 'Incidence of mycotoxigenic *Penicillia* in feeds of Andhra Pradesh, India' *International Journal of Biotechnology and Molecular Biology Research*, 2(2), pp.46-50.

r-Biopharm RIDASCREEN ®Aflatoxin M1, 2020, Art. No. R1121 Cheese (2015), 2. Available online: https://food.r-biopharm.com/products/ridascreen-aflatoxin-m1/ (Accessed in 2022)

Reddy, B.N. and Raghavender, C.R., (2007) 'Outbreaks of aflatoxicoses in India', *African Journal of Food, Agriculture, Nutrition and Development*, 7(5), pp. 1-16.

Schatzmayr, G., *et al*., (2006) 'Microbiologicals for deactivating mycotoxins', *Molecular Nutrition & Food Research*, 50(6), pp.543-551.

Simpson, D.R., Weston, G.E., Turner, J.A., Jennings, P. and Nicholson, P., (2001) 'Differential control of head blight pathogens of wheat by fungicides and consequences for mycotoxin contamination of grain', *European Journal of Plant Pathology*, 107(4), pp.421-431.

Sheikh-Ali, S.I., *et al*., (2014) 'The potential hazards of Aspergillus sp. in foods and feeds, and the role of biological treatment: A review', *Journal of Microbiology*, 52(10), pp.807-818.

Singh, D., *et al*., (2015) 'Molecular characterisation of Aspergillus flavus isolates from peanut fields in India using AFLP', *Brazilian Journal of Microbiology*, 46, pp.673-682.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., (2013) 'MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0', *Molecular Biology and Evolution*, 30(12), pp.2725-2729.

Tandon, B.N., *et al*., (1977) 'Study of an epidemic of jaundice, presumably due to toxic hepatitis, in northwest India', *Gastroenterology*, 72(3), pp.488-494.

Umesha, S., *et al*., (2017) 'Aflatoxins and food pathogens: impact of biologically active aflatoxins and their control strategies', *Journal of the Science of Food and Agriculture*, 97(6), pp.1698-1707.

Variane, A.C.F., *et al*., (2018) 'The occurrence of aflatoxigenic Aspergillus spp. in dairy cattle feed in Southern Brazil', *Brazilian journal of microbiology*, 49, pp.919-928.

Vaz, A., Cabral Silva, A.C., Rodrigues, P. and Venâncio, A., (2020) 'Detection methods for aflatoxin M1 in dairy products', *Microorganisms*, 8(2), pp. 246-262.

Vidyasagar. A., (2016) 'Facts About the Fungus Among Us**',** *Live Science*, Available at: https://www.livescience.com/53618-fungus.html (Accessed on: 12 November 2021)

World Health Organization, Department of Food Safety and Zoonoses, 2018 https://www.who.int/foodsafety/FSDigest_Aflatoxins_EN.pdf

Wu, F., Narrod, C., Tiongco, M. and Liu, Y., 'THE HEALTH ECONOMICS OF AFLATOXIN: GLOBAL BURDEN OF DISEASES', *Aflatoxin Control Project, IFPRI*, Washington DC, Working Paper 4.

Yu, J., Bhatnagar, D. and Ehrlich, K.C., (2002) 'Aflatoxin Biosynthesis', *Revista iberoamericana de micología*, 19(4), pp.191-200.